Molecular Therapy
Methods & Clinical Development
Protocol

Genetic engineering of human and mouse CD4+ and CD8+ Tregs using lentiviral vectors encoding chimeric antigen receptors

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The last decade has seen a significant increase of cell therapy protocols using effector T cells (Teffs) in particular, but also, more recently, non-engineered and expanded polyclonal regulatory T cells (Tregs) to control pathological immune responses such as cancer, autoimmune diseases, or transplantation rejection. However, limitations, such as stability, migration, and specificity of the cell products, have been seen. Thus, genetic engineering of these cell subsets is expected to provide the next generation of T cell therapy products. Lentiviral vectors are commonly used to modify Teffs; however, Tregs are more sensitive to mechanical stress and require specific culture conditions. Also, there is a lack of reproducible and efficient protocols to expand and genetically modify Tregs without affecting their growth and function. Due to smaller number of cells and poorer viability upon culture *in vitro*, mouse Tregs are more difficult to transduce and amplify *in vitro* than human Tregs. Here we propose a step-by-step protocol to produce both human and mouse genetically modified CD8+ and CD4+ Tregs in sufficient amounts to assess their therapeutic efficacy in humanized immunocompromised mouse models and murine models of disease and to establish pre-clinical proofs of concept. We report, for the first time, an efficient and reproducible method to isolate Tregs from human blood or mouse spleen, transduce with a lentiviral vector, and culture, in parallel, CD8+ and CD4+ Tregs while preserving their function. Beyond chimeric antigen receptor (CAR)-Treg cell therapy, this protocol will promote the development of potential new engineered T cell therapies to treat autoimmune diseases and transplantation rejection.

INTRODUCTION

Regulatory T cells (Tregs) are used as cell therapy to treat autoimmune diseases and control allogeneic immune responses in transplantation rejection. Deep knowledge of Treg biology acquired over decades has highlighted potential targets and strategies to redirect cell therapy specificity and function using genetic engineering.[1] Lentiviral vectors, rather than retroviral vectors, are interesting tools that enable stable integration and further expression of a transgene in T cells.[2] Genetic modifications are currently used to restore, stabilize, improve, or convert T cell functions. For example, FOXP3 ectopic expression in CD4+ effector T cells (Teffs) was used in human and mouse cells to obtain Tregs.[2–4] T cell receptor (TCR) gene transfer using lentiviral vectors showed promising results for islet-specific CD4+ Tregs to control type 1 diabetes[5] and for tumor-antigen-specific CD8+ Teffs to control melanoma development.[6] Development of the chimeric antigen receptor (CAR) technology made T cell engineering more attractive to treat cancer,[7] autoimmune diseases, and transplant rejection in patients.[8] CAR CD4+ Tregs were recently generated to control experimental autoimmune encephalitis (EAE), as a model of multiple sclerosis, and human leukocyte antigen (HLA)-mismatched graft rejection.[9–12] We highlighted the potential of polyclonal CD8+ Treg cell therapy in models of xenogeneic graft-versus-host disease (GVHD) and human skin transplantation rejection in immune humanized immunodeficient mice.[13–15] We recently described that conferring a specificity to CD8+ Tregs toward graft donor HLA by lentiviral delivery of a CAR greatly improved control of solid organ transplant rejection and GVHD occurrence in immune humanized mouse models.[16] Besides, CD8+ Tregs were shown to control memory responses more efficiently than CD4+ Tregs, while CD4+ Tregs were more efficient than CD8+ Tregs at controlling the naive immune response,[17] and synergy between CD4+ and CD8+ Tregs is likely.[18] Thus, there is a huge potential to optimize and enhance both CD4+ and CD8+ Treg functionality for novel cell therapies.

While studies reported 60% to 85% success in genetic engineering of human T cells and 20% to 40% for human CD4+ Tregs using vesicular stomatitis virus G (VSVg)-pseudotyped lentiviral vectors,[5,11,19] we faced the lack of clear protocol to manufacture genetically modified human and mouse Tregs and, particularly, CD8+ Tregs in sufficient quantity to assess their safety and therapeutic efficacy. The use of rodent models and, in particular, immune humanized immunodeficient

Received 29 June 2020; accepted 10 November 2020;
https://doi.org/10.1016/j.omtm.2020.11.008.

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rat and mouse models are key to establish pre-clinical proof of concept of engineered Treg therapy candidates before clinical investigation in humans can be envisioned.[20]

Here we describe an efficient and reproducible protocol to generate large amount of genetically engineered human and mouse CD4$^+$ and CD8$^+$ Tregs using VSVg- or murine leukemia virus (MLV)-pseudotyped lentiviral vectors, respectively, in less than 2 weeks. The lentiviral vectors encoding for CARs were produced following protocols that have been already largely described.[21] The protocol that is described here is composed of two parts: the generation of genetically modified CD8$^+$ and CD4$^+$ Tregs from 1) human blood and 2) mouse spleen, from their isolation to their transduction and expansion. This protocol can also be used for human or mouse Treg isolation and culture without intention of genetic modification.

This protocol is highly relevant to investigate cell therapy candidates in various disease areas, such as genetic and autoimmune diseases, transplantation, and cancer, as well as for fundamental research to decipher the role of new molecules in T cell biology and function. Biosafety level S2 (BSL-2) and cell sorter equipments and flow cytometry competences are required to apply this protocol and can represent potential limitations.

MATERIALS

Reagents

- Human blood (Etablissement Français du Sang, Nantes, France)
- Mouse (C57BL/6J, Janvier Labs, 8- to 16 weeks old)
- HLA-A*02-specific CAR (A2-CAR) lentiviral vector made with pMD2.G VSVg-envelope plasmid (Addgene #12259), Rous sarcoma virus promoter (pRSV)-Rev (Addgene #12253), and pMDLg/pRRE (Addgene #12251) packaging plasmids containing central polypurine tract (cPPT), WPRE, and inactivated 3′LTR (long terminal repeat) and an entry plasmid vector containing the gene of interest (A2-CAR) and the truncated gene encoding for the low-affinity nerve growth factor receptor (ΔNGFR [nerve growth factor receptor]) as a reporter marker
- Myelin oligodendrocyte glycoprotein (MOG)-CAR lentiviral vector made with human cytomegalovirus promoter (pHCMV)-EcoEnv MLV-envelope plasmid (Addgene #15802), pRSV-Rev (Addgene #12253) and pMDLg/pRRE (Addgene #12251) packaging plasmids containing cPPT, Woodchuck hepatitis virus (WHP) posttranscriptional regulatory element (WPRE), and inactivated 3′LTR and an entry plasmid vector containing the gene of interest and a ΔNGFR as a reporter marker
- Phosphate buffered saline (PBS) 1× (Gibco #14190-144)
- RPMI 1640 medium (Gibco #31870-025)
- Heat-inactivated fetal bovine serum (FBS) (Thermo Fischer Scientific #10270-106)
- Human AB serum (Sigma #H4522, 100 mL)
- Penicillin (10,000 U/mL)/streptomycin (10 mg/mL) 100× (Gibco #15140-122)
- Sodium pyruvate 100 mM 100× (Thermo Fisher Scientific #11360-039)
- L-glutamine (Sigma-Aldrich #G1251, 100 g)
- HEPES buffer (Gibco #15630-056, 1 M)
- Minimum essential media (MEM) non-essential amino acids 100× (Thermo Fisher Scientific #11140-035)
- β-mercaptoethanol (Sigma #M3148)
- Rapamycin (Rapamune) (Pfizer)
- Ethylenediaminetetraacetic acid (EDTA) (Sigma #200-449-4)
- Sterile H_2O
- Ficoll (Eurobio #CMSMSL01-01)
- Anti-mouse CD3 / anti-mouse CD28 monoclonal antibody (mAb)-coated beads (Gibco #11452D)
- DAPI (Invitrogen #D3571)
- Anti-mouse immunoglobulin G (IgG) Dynabeads (Invitrogen #11033)
- OneComp eBeads (eBioscience #01-1111-42)
- Human interleukin-2 (IL-2) (Proleukin, Novartis)
- Mouse IL-2 (PeproTech #212-12)
- Human IL-15 (Miltenyi Biotec #130-095-700)
- Mouse IL-15 (PeproTech #210-15)
- Human IL-7 (Miltenyi Biotec # 130-095-367)
- Protranduzin A (PTDA) (JPT #TE-PTDB-3)
- Bleach solution
- 96-well flat bottom plates (BD Falcon #353072)
- 15 mL and 50 mL conical centrifuge tubes (BD Falcon #227261 and #188271)
- 1.5 mL Eppendorf microtubes (VWR #211-0015)
- 5 mL, 10 mL, and 25 mL sterile serological pipettes (Thermo Fisher Scientific or equivalent)
- Sterile low retention universal tips with filter (Grosseron or equivalent)
- Woven mesh filters Nitex (VWR #SEFA03-60/35)
- Syringe
- 100 μm cell strainer (BD Falcon #352360)
- 500 mL Corning filter unit 0.22 μm nitrocellulose membranes (Dutscher #430758)
- Monoclonal antibodies (mAbs) (Table 1)

Equipment

- Centrifuge
- Laminar flow hood
- Incubator for cell culture
- Flow cytometer
- Fluorescence-activated cell sorting (FACS) Aria cell sorter (BD Biosciences or equivalent)
- Inverted fluorescence microscope (if green fluorescence protein [GFP] reporter marker is used)
- Micropipettes (Gilson type or equivalent)
- Magnet (DynaMag 50, ThermoFisher Scientific #12302D or equivalent)
- Vortexer

Reagent setup

Staining buffer

1 L PBS 1× + 20 mL FBS (2%) + 5 mL EDTA 1 M (5 mM).

Table 1. mAbs used for T cell sorting

Monoclonal antibody	Provider	Clone	Fluorochrome
Anti-human CD19 mAb	BD Biosciences	HIB19	purified
Anti-human CD14 mAb	BD Biosciences	M5E2	purified
Anti-human CD16 mAb	BD Biosciences	3G8	purified
Anti-human CD3 mAb	BD Biosciences	SK7	PeCy7
Anti-human CD3 mAb	European Collection of Cell Culture	OKT3	purified
Anti-human CD8 mAb	BD Biosciences	RPA-T8	APC
Anti-human CD25 mAb	BD Biosciences	M-A251	APC-Cy7
Anti-human CD127 mAb	BD Biosciences	hIL-7R-M21	PE
Anti-human CD4 mAb	BD Biosciences	RPA-T4	PerCPCy5,5
Anti-human CD45RC mAb	IqProduct	MT2	FITC
Anti-human CD28 mAb	European Collection of Cell Culture	CD28.2	purified
Anti-human CD56 mAb	BD Biosciences	MY31	purified
Anti-LNGFR mAb	Miltenyi Biotec	REA844	PeCy7
Anti-mouse CD4 mAb	BD Biosciences	RM4-5	PeCy7
Anti-mouse CD8 mAb	BD Biosciences	53-6.7	APC
Anti-mouse CD45RC mAb	BD Biosciences	DNL-1.9	PE
Anti-mouse CD25 mAb	BD Biosciences	PC61	PerCPCy5,5
Anti-mouse CD11c mAb	BD Biosciences	N418	BV421
Anti-mouse NK1.1 mAb	BD Biosciences	PK136	V450
Anti-mouse CD19 mAb	BD Biosciences	1D3	purified
Anti-mouse CD49b mAb	BD Biosciences	DX5	purified
Anti-mouse CD11b mAb	BD Biosciences	M1/70	purified

Human T cell culture medium

500 mL RPMI 1640 medium + 5 mL penicillin/streptomycin 100× + 5 mL sodium pyruvate (1 mM) + 5 mL L-glutamine (1 mM) + 5 mL HEPES buffer (10 mM) + 5 mL MEM non-essential amino acids 100×, supplemented with 5% (before transduction) or 10% (after transduction) human AB serum, IL-2 (100 U/mL for Teffs and 1,000 U/mL for Tregs), IL-15 (5 ng/mL for Teffs and 10 ng/mL for CD8$^+$ Tregs), IL-7 (5 ng/mL for Teffs), and rapamycin (50 nM for Tregs only).

Mouse T cell culture medium

500 mL RPMI 1640 medium + 5 mL penicillin/streptomycin 100× + 5 mL sodium pyruvate (1 mM) + 5 mL L-glutamine (1 mM) + 5 mL HEPES buffer (10 mM) + 5 mL MEM non-essential amino acids 100× + 50 μM β-mercaptoethanol, supplemented with 10% FBS, IL-2 (1,000 U/mL), IL-15 (20 U/mL for CD8$^+$ T cells only), and rapamycin (50 nM for Tregs only).

Hypotonic solution for red blood cell lysis

8.29 g NH_4Cl + 1 g $KHCO_3$ + 37.2 mg Na2 EDTA + 900 mL distilled H_2O. Adjust the pH to 7.0 with 10 N NaOH. Complete to 1 L with sterile distilled H_2O. Filter through 0.22 μm nitrocellulose membrane.

PROCEDURE

NOTE: All of these protocols must be performed in a sterile manner. Experiments with human cells and lentiviral vectors need to be performed in a BSL-2 lab.

NOTE: All the liquid and solid materials in contact with the viral particles must be disinfected in a 10% bleach bath for at least 12 h. The single-use material is then thrown into a Déchets d'Activités de Soin à Risques Infectieux (DASRI) bin for incineration.

Genetic modification in primary human T cells using lentiviral vectors

Sorting of human Tregs from blood

NOTE: Using this protocol, approximately 50,000 CD8$^+$ Tregs/mL and 12,500 CD4$^+$ Tregs/mL blood are isolated. 50 mL blood is sufficient to generate 10^7 and 2.5 × 10^6 genetically modified CD8$^+$ and CD4$^+$ Tregs, respectively.

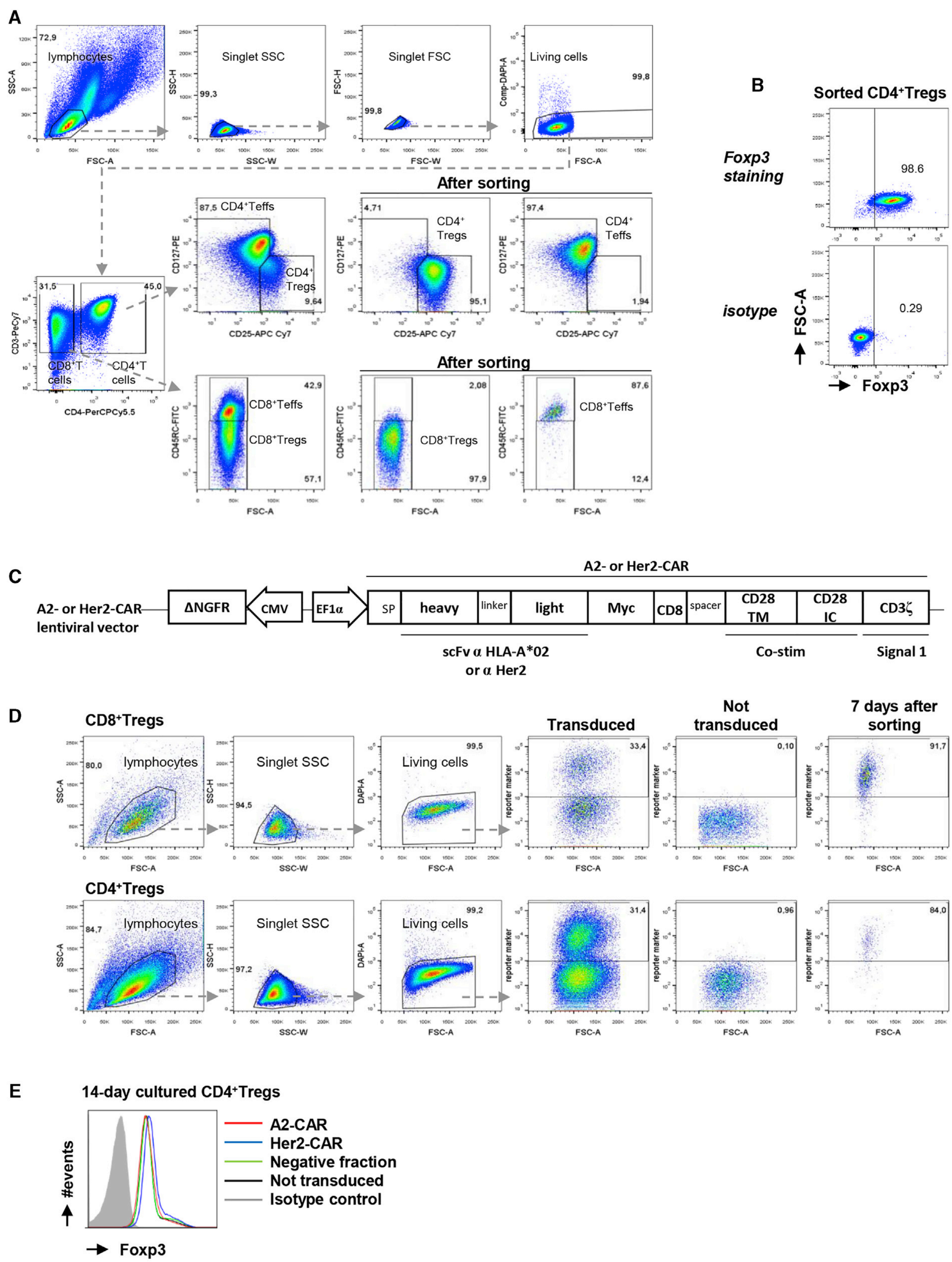
1. Harvest human peripheral blood mononuclear cells (PBMCs) by Ficoll gradient centrifugation. Dilute blood at 0.5× with PBS 1×. Gently add 30 mL diluted blood on the top of a 15 mL Ficoll solution. Centrifuge for 20 min at 770 *g*, 20°C, with moderate acceleration (7/9) and no braking (0).
2. Delicately harvest the ring of PBMCs floating on the Ficoll phase and wash the cells with 50 mL PBS 1× and a 10 min 600 *g* centrifugation. Discard the supernatant.
3. To eliminate red blood cells and platelets, resuspend and pool the PBMC pellets in 10 mL hypotonic solution, incubate 5 min at room temperature (RT), then wash with 50 mL PBS 1× and a 10 min 185 *g* centrifugation. Discard the supernatant.

NOTE: Transduction of T cells isolated from thawed PBMCs is also possible.

4. Count the cells to adjust cell concentration to 2 × 10^8 cells/mL in staining buffer.

NOTE: 2 × 10^6 PBMCs/mL blood should be isolated.

NOTE: To isolate Tregs from PBMCs, enrichment before sorting by negative selection is recommended from steps 5–9.



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5. Stain unwanted with purified antibodies specific for B cells (anti-CD19 mAb, HIB19 clone), natural killer (NK) cells (anti-CD56 mAb, MY31 clone), and monocytes (anti-CD14 and anti-CD16 mAbs, M5E2 and 3G8 clones, respectively) (Table 1) diluted at 10 μg/mL in staining buffer.
6. Add 50 mL staining buffer and centrifuge for 10 min at 430 *g* to wash the cells. Discard the supernatant.
7. Collect 3.5 μL anti-mouse IgG-coated beads (i.e., 1.4×10^6 beads) for 10^6 PBMCs. Wash anti-mouse IgG-coated beads three times before use: add 100 vol of staining buffer onto the beads (350 μL for 3.5 μL beads), place on the magnet for 1 min, and discard the supernatant. Repeat twice. Resuspend beads in 10 vol of staining buffer.

NOTE: For 50 mL blood or 10^8 PBMCs, 350 μL anti-mouse IgG-coated beads (i.e., 1.4×10^8 beads) are washed three times with 35 mL staining buffer and resuspended in 3.5 mL staining buffer.

8. Mix the PBMC pellet with the diluted anti-mouse IgG-coated beads and incubate for 10 min at 4°C under gentle agitation. Then place the tube on the magnet for 1 min and transfer the supernatant in a new tube. Unwanted cells are retained on the magnet. Place the new tube on the magnet and repeat the procedure twice to make sure all unwanted cells and beads are removed.
9. Count the cells. Adjust cell concentration to 2×10^8 cells/mL in staining buffer.

NOTE: 50% to 70% of cells are expected to be removed. When starting with 50 mL blood, 3 to 5×10^7 cells should remain.

10. Stain the cells for FACS sorting depending on the subset required (Table 1; Figure 1A).
 a. To sort total CD4$^+$ and/or CD8$^+$ T cells, add anti-CD3 (SK7 clone), anti-CD4 (RPA-T4 clone), and anti-CD8 (RPA-T8 clone) mAbs.
 b. To sort CD4$^+$ Tregs, add anti-CD3 (SK7 clone), anti-CD4 (RPA-T4 clone), anti-CD25 (M-A251 clone), and anti-CD127 (hIL-7R-M21 clone) mAbs.
 c. To sort CD8$^+$ Tregs, add anti-CD3 (SK7 clone), anti-CD8 (RPA-T8 clone), and anti-CD45RC (MT2 clone) mAbs, as previously described.[15,22,23]

NOTE: If CD56$^+$ cells were not depleted before sorting, add anti-CD56 (MY31 clone) mAb.

 d. To sort both CD4$^+$ and CD8$^+$ Tregs, add anti-CD3 (SK7 clone), anti-CD4 (RPA-T4 clone), anti-CD25 (M-A251 clone), anti-CD127 (hIL-7R-M21 clone), and anti-CD45RC (MT2 clone) mAbs (Figure 1A).

NOTE: If CD56$^+$ cells were not depleted before sorting, add anti-CD56 (MY31 clone) mAb.

Incubate the cells with mAbs for 15 to 30 min at 4°C.

NOTE: We recommend a full 30 min incubation when performing CD25 staining for good flow cytometry detection. In the absence of CD25 staining, 15 min incubation is sufficient.

NOTE: Anti-CD4 and anti-CD8 mAbs must be concomitantly added to eliminate CD4$^+$CD8$^+$ T cells that represent 2% of blood T cells.

NOTE: Label beads (Compbeads) with each single mAb to set up a compensation matrix for further processing in flow cytometry.

11. Add 50 mL staining buffer to the cell suspension, centrifuge 10 min at 430 *g* 4°C and discard the supernatant.
12. Resuspend the cell pellet at 6×10^7 cells/mL in staining buffer, filter on a 60 μm tissue, and label dead cells by adding 0.1 μg/mL DAPI.
13. Sort cells with a 70 μm nozzle cell sorter by gating on lymphocyte morphology (low FSC-A and SSC-A; Figure 1A) and one of the following:
 a. DAPI$^-$CD3$^+$CD4$^-$CD8$^+$ cells to sort total CD8$^+$ T cells
 b. DAPI$^-$CD3$^+$CD4$^+$CD8$^-$ cells to sort total CD4$^+$ T cells
 c. DAPI$^-$CD3$^+$CD4$^+$CD25^{high}CD127$^{low/-}$ cells to sort CD4$^+$ Tregs
 d. DAPI$^-$CD3$^+$CD8$^+$ (or CD4$^-$) CD45RC$^{low/-}$ (CD56$^-$) cells to sort CD8$^+$ Tregs

NOTE: CD4$^+$ and CD8$^+$ Teffs and Tregs can be simultaneously sorted using four-way sorting tubes in FACS Aria II (BD Biosciences).

NOTE: More than 95% of sorted CD4$^+$ Tregs express FOXP3 after sorting (Figure 1B).

Figure 1. Gating strategy for human Treg and Teff isolation and genetically modified T cell purification

(A) Human CD4$^+$ and CD8$^+$ Tregs and Teffs were sorted from T cell-enriched PBMCs by FACS Aria by gating on small morphology (Side Scatter - Area [SSC-A]/Forward Scatter - Area [FSC-A]), SSC and FSC singlets, DAPI$^-$ living cells, and CD3$^+$ cells. CD4$^+$ T cells were sorted on CD127$^{low/-}$ CD25$^+$ expression as CD4$^+$ Tregs and on an inverted gate as CD4$^+$ Teffs. CD4$^-$ T cells were sorted on CD45RC^{high} or CD45RC$^{low/-}$ expression as CD8$^+$ Teffs and CD8$^+$ Tregs, respectively. Cell purity after sorting is shown. The frequency of each cell subsets in the previous cell selection is indicated. (B) Flow cytometry staining of FOXP3 in human CD4$^+$ CD25$^+$ CD127$^{low/-}$ T cells after FACS Aria sorting. Isotype staining is shown on the bottom. Representative staining of two experiments. (C) Schematic of the second generation of CAR used. Sequences for the transmembrane CD28 and intracellular CD28 and CD3ζ signaling portions were placed C-terminal to the anti-HLA-A*02- or Her2-specific scFv, a c-Myc epitope tag, and a CD8α stalk sequence under an EF1α promotor. The full CAR construct was cloned into a bi-directional lentiviral vector encoding ΔNGFR as a transduction marker under a CMV promotor. (D) Left: human CD8$^+$ Tregs (top) and CD4$^+$ Tregs (bottom) were sorted on reporter marker expression (here we used ΔNGFR) after gating on morphology, singlet cells, and DAPI$^-$ living cells on day 7. Middle: untransduced cells are shown as negative control of staining. Right: expression of the reporter marker on day 14 (i.e., 7 days after sorting on ΔNGFR). (E) Flow cytometry staining of FOXP3 marker in HLA-A*02-CAR (red line), Her2-CAR (blue line), negative fraction of cell sorted on day 7 (green line), and not transduced (black line) CD4$^+$ Tregs after 14-day culture. Isotype control staining is shown in filled gray.

14. Wash the collected cells twice with 50 mL staining buffer and 430 *g* centrifugation for 10 min at 4°C.
15. Count the cells. Adjust cell concentration to 1 × 10^6 cells/mL in human T cell culture medium supplemented with 5% human AB serum and cytokines: 1,000 U/mL IL-2 for $CD4^+$ Tregs; 1,000 U/mL IL-2 and 10 ng/mL IL-15 for $CD8^+$ Tregs; 100 U/mL IL-2, 5 ng/mL IL-15, and 5 ng/mL IL-7 for Teffs.

NOTE. A high concentration of human AB serum partially inhibits cell transduction.

Lentiviral transduction of human T cells

1. Add 50 μL/well in a 96-well flat bottom plate of 1 μg/mL anti-CD3 (OKT3 clone) mAb diluted in PBS 1× and incubate for ≥1 h at 37°C.

NOTE: Low-dose (1 μg/mL) anti-CD3 mAbs is preferred for T cell transduction rate and growth.

2. Remove free mAbs by adding 100 μL/well PBS 1×, centrifuging for 1 min at 430 *g*, and discarding supernatant. Repeat twice.

CAUTION: Do not allow the coated plastic to dry; discard the PBS 1× of the final wash when cells are ready to be seeded.

3. Add 1 μg/mL anti-CD28 (CD28.2 clone) mAb in T cell suspension and seed 10^5 cells (100 μL) per well in the anti-CD3 mAb plastic-coated plate. Promote T cell activation by a 1 min 430 *g* spin. Incubate the cells overnight (ON) at 37°C 5% CO_2.

NOTE: Polyclonal stimulation of $CD8^+$ Tregs after sorting and ≥12 h before transduction is critical for transduction rate.

NOTE: Beads can be used instead of purified anti-CD3 and anti-CD28 mAbs at a 1:1 bead:cell ratio.

4. On day +1, gently add lentiviral vector at a multiplicity of infection (MOI) (i.e., the number of transducing units [TU] of viral particles/cell) 10 (i.e., 10^6 TU for 10^5 cells; i.e., 10 μL of a 10^8 TU/mL batch) on the top middle of each well, taking care not to disturb T cell clusters. Ensure total lentiviral vector penetration by a 1 min 430 *g* spin. Incubate the cells for 24 h at 37°C 5% CO_2.

NOTE: For a first transduction with a new lentiviral vector, we recommend testing a range of lentiviral vector doses to assess maximal transduction rate and lentiviral vector toxicity.

NOTE: Keep some cells free of virus as negative control of transduction for flow cytometry staining or functional assay.

5. On day +2, repeat step 4.

NOTE: GFP should be visible by microscopy within 48 h after first transduction.

NOTE: One additional step of lentiviral transduction can significantly improve the transduction rate and yield of transduced $CD4^+$ Tregs, but not $CD8^+$ Tregs.

6. One day after the last transduction (i.e., day +3 if two transductions were performed on $CD8^+$ Tregs and day +4 if three transductions were performed on $CD4^+$ Tregs), enrich culture medium for higher cell expansion by adding 100 μL/well of T cell culture medium supplemented with 15% human AB serum and 2× cytokines: 2,000 U/mL IL-2 for $CD4^+$ Tregs; 2,000 U/mL IL-2 and 20 ng/mL IL-15 for $CD8^+$ Tregs; and 200 U/mL IL-2, 10 ng/mL IL-15, and 10 ng/mL IL-7 for Teffs. Incubate the cells at 37°C 5% CO_2.
7. We assume total consumption of cytokines in 48 h, thus supplement T cell culture with fresh cytokines every 2 days by adding 20 μL/well culture medium supplemented with 10× cytokines: 10,000 U/mL IL-2 for $CD4^+$ Tregs; 10,000 U/mL IL-2 and 100 ng/mL IL-15 for $CD8^+$ Tregs; and 1,000 U/mL IL-2, 50 ng/mL IL-15, and 50 ng/mL IL-7 for Teffs.
8. High cell proliferation can require addition of culture medium and splitting of cells. If the culture medium turns orange-yellow, mix gently the cell suspension, transfer 100 μL of T cell suspension in a new well, and add 100 μL/well of culture medium supplemented with 2× cytokines: 2,000 U/mL IL-2 for $CD4^+$ Tregs; 2,000 U/mL IL-2 and 20 ng/mL IL-15 for $CD8^+$ Tregs; and 200 U/mL IL-2, 10 ng/mL IL-15, and 10 ng/mL IL-7 for Teffs.

NOTE: Progressive splits are preferred; cell density is critical for their survival.

Selection and expansion of transduced cells

NOTE: Isolation of living transduced cells is possible if a surface cell membrane reporter marker, like ΔNGFR, or a fluorescent reporter marker has been included in the entry plasmid construct (Figure 1C).

NOTE: For research use only, a fluorescent reporter marker such as GFP, cyan fluorescent protein (CFP), or yellow fluorescent protein (YFP) is preferred to avoid the flow cytometry staining step of the reporter marker. For clinical application, we recommend using the ΔNGFR reporter marker (as discussed below).

1. On day +7, harvest cells and wash thoroughly with 50 mL staining buffer by a 10 min 430 *g* centrifugation. Discard the supernatant.

NOTE: If the plasmid construct includes a fluorescent reporter marker such as GFP, go to step 3 below. If no fluorescent but a membrane reporter marker is intended, perform a flow cytometry staining by following step 2 below.

NOTE: The reporter marker ΔNGFR is used as an example for the genetically modified cell FACS sorting (Figure 1D).

2. Stain the cells with 2 μg/mL anti-ΔNGFR (or equivalent) mAb for 15 min at 4°C, then wash the cells with 50 mL staining buffer and a 10 min 430 *g* centrifugation. Discard the supernatant.

3. Adjust cell concentration to 6×10^7 cells/mL in a minimum volume of 200 μL staining buffer. Filter the cells on a 60 μm filter and label dead cells by adding 0.1 μg/mL DAPI.
4. Sort cells with a 70 μm nozzle cell sorter by gating on $DAPI^-\Delta NGFR^+$ or equivalent reporter $marker^+$ cells (Figures 1C and 1D).
5. Wash the collected cells twice with 50 mL staining buffer.
6. Count cells and adjust cell concentration to 2.5×10^5 cells/mL in T cell culture medium supplemented with 10% of human AB serum, and 1× cytokines: 1,000 U/mL IL-2 for $CD4^+$ Tregs; 1,000 U/mL IL-2 and 10 ng/mL IL-15 for $CD8^+$ Tregs; and 100 U/mL IL-2, 5 ng/mL IL-15, and 5 ng/mL IL-7 for Teffs.
7. Stimulate the sorted cells by coating a 96-well flat bottom plate with 50 μL/well of 1 μg/mL anti-CD3 (OKT3 clone) mAb diluted in PBS 1×. Incubate for ≥1 h at 37°C.
8. Remove free mAbs by washing gently with 100 μL/well PBS 1×, centrifuging for 1 min at 430 *g* 4°C and discarding supernatant. Repeat twice.

CAUTION: Do not let the coated plastic dry; discard the PBS 1× of the final wash when cells are ready to be seeded.

9. Add 1 μg/mL anti-CD28 (CD28.2 clone) mAb to the T cell solution and seed 5×10^4 cells (200 μL) per well in the anti-CD3 mAb-coated plastic plate. Promote T cell activation by a 1 min 430 *g* spin. Incubate the cells at 37°C 5% CO_2.
10. We assume total consumption of cytokines in 48 h, thus supplement T cell culture with fresh cytokines every 2 days by adding 20 μL/well T cell culture medium supplemented with 10× cytokines: 10,000 U/mL IL-2 for $CD4^+$ Tregs; 10,000 U/mL IL-2 and 100 ng/mL IL-15 for $CD8^+$ Tregs; and 1,000 U/mL IL-2, 50 ng/mL IL-15, and 50 ng/mL IL-7 for Teffs.
11. When the culture medium turns orange-yellow due to high cell proliferation, likely on days +11 and +13, gently mix the cell suspension, transfer 100 μL of T cell suspension in a new well, and add 100 μL/well of culture medium supplemented with 2× cytokines: 2,000 U/mL IL-2 for $CD4^+$ Tregs; 2,000 U/mL IL-2 and 20 ng/mL IL-15 for $CD8^+$ Tregs; and 200 U/mL IL-2, 10 ng/mL IL-15, and 10 ng/mL IL-7 for Teffs.

NOTE: Progressive splits are preferred; cell density is critical for T cell survival.

NOTE: FOXP3 is still expressed in $CD4^+$ Tregs after 14 days of culture (Figure 1E).

NOTE: Cells can be expanded for up to 1 month when stimulated with anti-CD3 and anti-CD28 mAbs every 7 days.[15]

Genetic modifications in primary mouse T cells using lentiviral vectors

Sorting of mouse Tregs from spleen

1. Harvest the spleen from an 8- to 16-week-old mouse and save it in cold PBS 1×.
2. Transfer the spleen into a 100 μm cell strainer placed onto a 50 mL falcon. Perfuse the spleen with 5 mL PBS 1×. Crush the spleen with a syringe's piston to dissociate the cells. Rinse the strainer with 10 mL PBS 1× and wash by completing to 50 mL with PBS 1× and centrifuging 10 min at 430 *g* 4°C. Discard the supernatant.
3. To eliminate red blood cells and platelets, resuspend the splenocyte pellet in 5 mL of hypotonic solution, incubate for 5 min at RT, then wash with 50 mL PBS 1× and a 10 min 430 *g* 4°C centrifugation. Discard the supernatant.
4. Count the cells to adjust cell concentration to 6×10^7 cells/mL in staining buffer.

NOTE: The number of splenocytes should be between 7×10^7 and 1×10^8 cells per animal.

NOTE: Spleens from several mice can be pooled to obtain more Tregs. If required, follow steps 4–9 using anti-CD19 (1D3 clone), anti-CD49b (DX5 clone), and anti-CD11b (M1/70 clone) purified mAbs to enrich T cells before sorting (Table 1).

5. Stain the cells for FACS sorting depending on the subset required (Table 1 and Figure 2A).
   a. To sort total $CD4^+$ and $CD8^+$ T cells, add anti-NK1.1 (PK136 clone), anti-CD11c (N418 clone), anti-CD4 (RM4-5 clone), and anti-CD8 (53-6.7 clone) mAbs.
   b. To sort $CD4^+$ Tregs, add anti-NK1.1 (PK136 clone), anti-CD11c (N418 clone), anti-CD4 (RM4-5 clone), and anti-CD25 (PC61 clone) mAbs.
   c. To sort $CD8^+$ Tregs, add anti-NK1.1 (PK136 clone), anti-CD11c (N418 clone), anti-CD8 (53-6.7 clone), and anti-CD45RC (DNL-1.9 clone) mAbs.
   d. To sort both $CD4^+$ and $CD8^+$ Tregs, add anti-NK1.1 (PK136 clone), anti-CD11c (N418 clone), anti-CD4 (RM4-5 clone), anti-CD25 (PC61 clone), anti-CD8 (53-6.7 clone), and anti-CD45RC (DNL-1.9 clone) mAbs (Figure 2A).

Incubate the cells with mAbs for 30 min at 4°C.

NOTE: Label beads (Compbeads) with each Ab to set up a compensation matrix for further processing in flow cytometry.

6. Wash the cells with 50 mL staining buffer with a 10 min 430 *g* 4°C centrifugation and discard the supernatant.
7. Resuspend cells at 6×10^7 cells/mL, filter on a 60 μm tissue, and label dead cells by adding DAPI at a final concentration of 0.1 μg/mL.
8. Sort cells with a 70 μm nozzle cell sorter by gating on lymphocyte morphology (low FSC-A and SSC-A, (Figure 2A) and on one of the following:
   a. $DAPI^-NK1.1^-CD11c^-CD8^+$ cells to sort total $CD8^+$ T cells
   b. $DAPI^-NK1.1^-CD11c^-CD4^+$ cells to sort total $CD4^+$ T cells
   c. $DAPI^-NK1.1^-CD11c^-CD8^-CD4^+CD25^{high}$ to sort $CD4^+$ Tregs
   d. $DAPI^-NK1.1^-CD11c^-CD4^-CD8^+CD45RC^-$ to sort $CD8^+$ Tregs.

NOTE: $CD4^+$ and $CD8^+$ Tregs and non-Tregs can be simultaneously sorted using a FACS Aria II with four-way sorting tubes (BD Biosciences) (Figure 2A).

NOTE: More than 90% sorted $CD4^+$ Tregs express FOXP3 after sorting (Figure 2B).

9. Wash the collected cells twice with 50 mL staining buffer and 10 min 430 *g* 4°C centrifugations.
10. Count the cells. Adjust cell concentration to 1×10^6 cells/mL in mouse T cell culture medium.

Lentiviral transduction of mouse T cells

1. Seed 10^5 cells (100 μL) per well in a 96-well flat-bottom plate.
2. Activate cells by adding anti-CD3 and anti-CD28 mAb-coated beads at a bead:cell ratio of 2:1. Incubate the cells ON at 37°C 5% CO_2.

CAUTION: From this step, experiments must be performed in a BSL-2 lab.

3. On day +1, gently remove half of the culture medium (50 μL). For each well, add 18 μg/mL PTDA and 10^6 TU (MOI 10) MLV-pseudotyped lentiviral vector in 250 μL final volume of culture medium, incubate for 10 min at 37°C, and then gently add the PTDA/lentiviral vector complex onto the 50 μL remaining cells. Centrifuge for 90 min at 1,000 *g* 32°C and incubate for 4 h at 37°C 5% CO_2.

NOTE: Final concentration of PTDA in 300 μL cell suspension is 15 μg/mL.

4. Centrifuge for 1 min at 430 *g*, discard the supernatant, and add 100 μL/well mouse T cell culture medium. Incubate for 6 days at 37°C 5% CO_2.

NOTE: We assume total consumption of cytokines in 48 h, thus fresh cytokines should be added every 2 days.

NOTE: High cell proliferation can require addition of culture medium and splitting of cells. If required, mix gently before splitting into two wells.

Timing

- Day 0
  - o Sort T cells: 4 h
- Day +1
  - o Transduce human T cells with lentiviral vector: 30 min
  - o Transduce mouse T cells with lentiviral vector: 6.5 h
- Day +2
  - o Transduced human T cells with lentiviral vector a 2^{nd} time: 30 min
- Day +3
  - o Enrich the culture medium: 30 min
- Day +7
  - o Isolate human transduced T cells: 2 h
  - o Assess human T cell function or expand them up to 3 more weeks
  - o Harvest mouse T cells and assess their function

Troubleshooting

Low transduction rate

Timing and level of transgene expression. GFP should be visible by microscopy within 2 days after the first transduction (Figures 3A and 3B). We observed a similar transduction rate in human $CD8^+$ Tregs (23.4% ± 1.9%) compared to $CD4^+$ Tregs (25.9% ± 3.7%) and total T cells (36.2% ± 7.3% and 28.2% ± 3.6% in $CD8^+$ and $CD4^+$ Teffs, respectively), whereas it was lower for mouse $CD8^+$ Tregs (49.5% ± 3.5%) compared to $CD4^+$ Tregs (91.6% ± 1.4%) (Figures 5E and 6G).

Dose. A range of lentiviral vector MOI should be tested to determine the optimal conditions of transduction for each cell type to preserve cell function and growth (Figures 3A, 3C, and 3D). No more than 2×10^7 TU/mL final lentiviral vector concentration for transduction is recommended to avoid toxicity.

Volume of reaction. The MOI is calculated as a ratio of lentiviral vector TU/cell but does not take into account the volume of reaction. Performing transduction in a small volume might improve virus-cell contact. However, reducing the volume of culture medium may affect cell growth.

Lentiviral vector design and storage. Lentiviral vector should be stored as single use aliquots and not refrozen. The choice of promotor, enhancer, and envelope protein may impact transduction efficiency and expression of the transgene.[24] The transduction rate depends on the expression cassette size since the longer >6 kbp reduces to 50% of the lentiviral vector titers.[24] For murine T cell transduction, an ecotropic viral envelope is required (Figure 6A).

Chemical and mechanical helpers. For murine T cell transduction, addition of protransduzin is required (Figures 6E and 6F). For human $CD8^+$ Tregs, we observed that addition of 8 μg/mL polybrene was toxic, while addition of 10 μg/mL protamine sulfate or 20 μg/mL plastic-coated retronectin[25] did not affect transduction rate or cell growth. In contrast to murine T cells (Figure 6D), centrifugation of human T cells after addition of the virus does not increase the transduction rate, but it may increase the number of transgene copies per cell and affect cell growth (Figure 5A).

Improving the purity of genetically modified cells. If the transduction rate is low, sorting of transduced cells can be required, depending on subsequent experiments. After purification by FACS Aria sorting, expression of the lentiviral vector-encoded protein remains stable up to 4 weeks in human Tregs.[16]

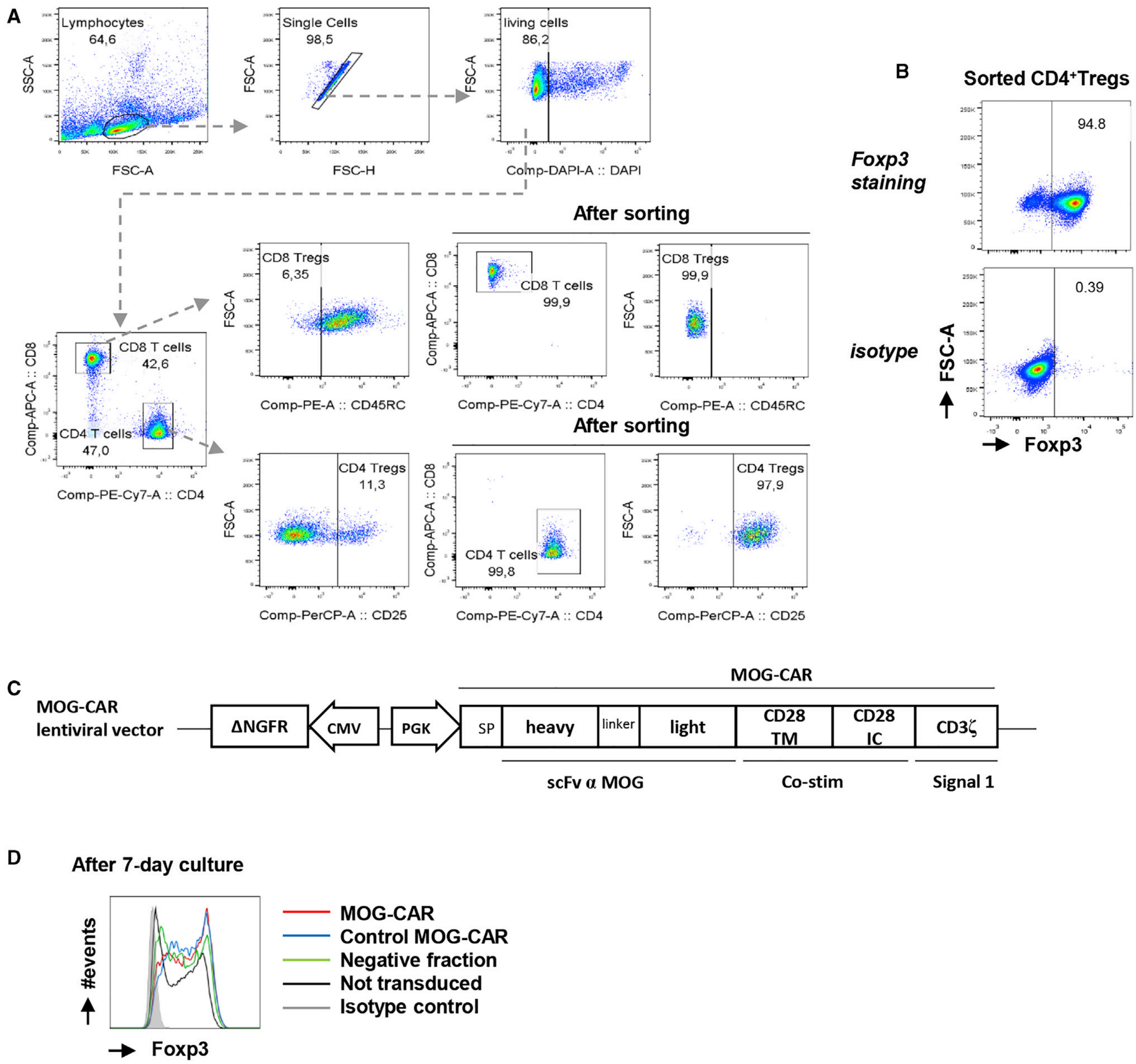

Figure 2. Mouse Treg and Teff isolation and characterization after transduction with a MOG-CAR recombinant lentiviral vector

(A) Mouse $CD4^+$ and $CD8^+$ Tregs and Teffs were sorted from splenocytes by FACS Aria by gating on small morphology (SSC-A/FSC-A), FSC singlets, $DAPI^-$ living $NK1.1^-CD11c^-$ cells. $CD4^+$ T cells were sorted on $CD4^+CD8^-$ expression, $CD4^+$ Tregs were sorted on $CD4^+CD8^-CD25^+$ expression, $CD8^+$ T cells were sorted on $CD8^+CD4^-$ expression, and $CD8^+$ Tregs were sorted on $CD8^+CD4^-CD45RC^-$ expression. Cell purity after sorting is shown. Numbers are the frequency of the cell subset in % of previous gate. (B) Flow cytometry staining of FOXP3 in murine $CD4^+$ $CD25^+$ $CD127^{low/-}$ T cells after FACS Aria sorting. Isotype staining is shown on the left. Representative staining of two experiments. (C) Schematic of the MOG-CAR vector. Sequences for the transmembrane CD28 and intracellular CD28 and CD3ζ signaling portions were placed C-terminal to the anti-MOG-specific scFv under a PGK promotor. The full CAR construct was cloned into a bi-directional lentiviral vector encoding ΔNGFR as a transduction marker under a CMV promotor. (D) Flow cytometry staining of FOXP3 marker in murine $CD4^+$ Tregs after 7-day culture and transduction or not (black line for untouched cells, green line for cells that have been in contact with the virus but not transduced) and with MOG-CAR (red line) or control CAR (blue line) lentiviral vectors.

Low cell growth

Culture medium. The choice of the culture medium is crucial for Treg expansion. RPMI, Xvivo-15, and Immunocult media are compatible with $CD4^+$ and $CD8^+$ Treg transduction and culture. FBS and human AB serum batches should be tested for their compatibility with T cell culture prior to use. Low dose serum is critical for human T cell transduction, but supplementation the day following transduction is beneficial for the cell growth (Figure 5B). Reducing

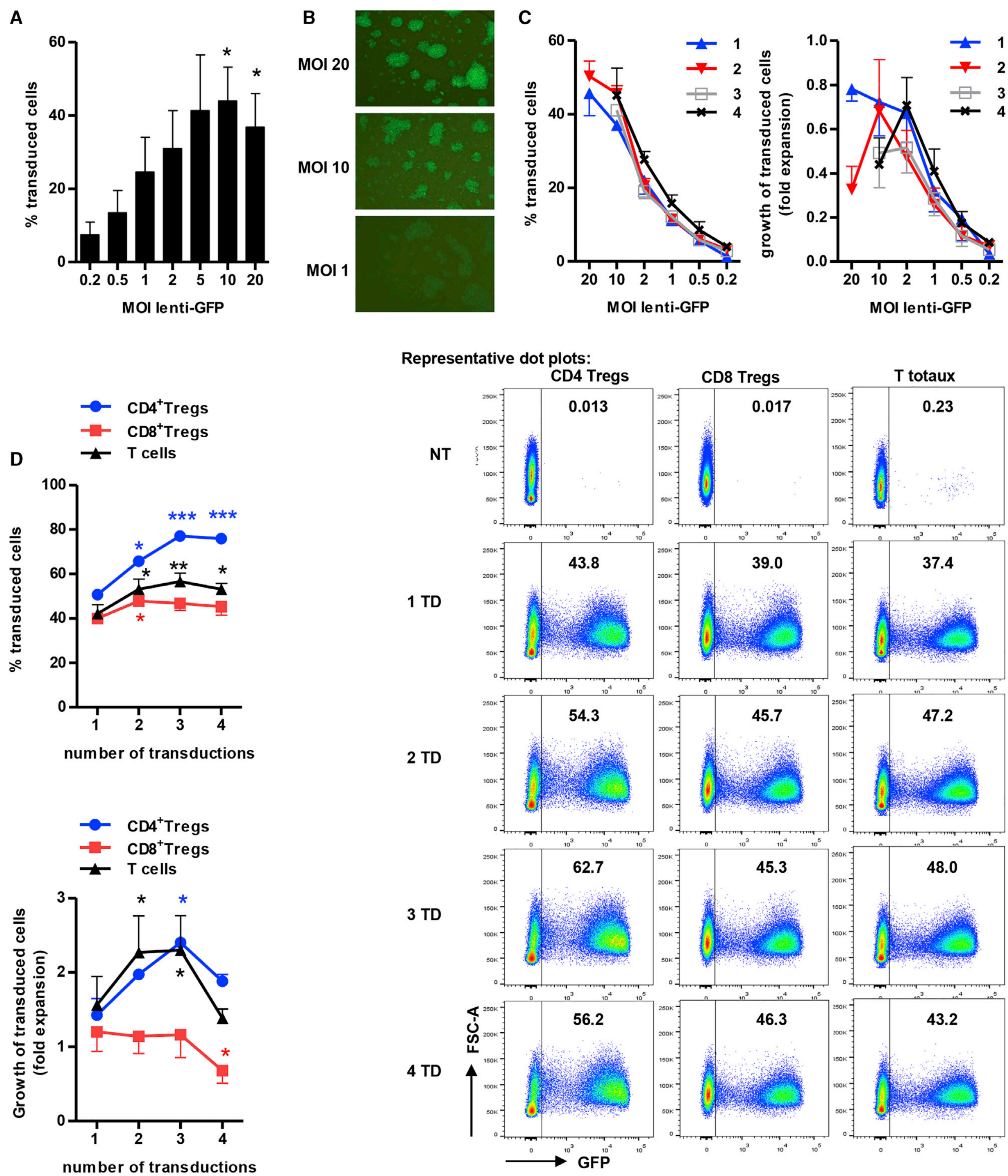

Figure 3. Determination of the optimal dose of lentiviral vector to obtain the maximum transduction rate in Tregs

(A and B) CD8+ Tregs were FACS Aria sorted, stimulated ON with anti-CD3 and anti-CD28 mAbs, transduced with a range of GFP-lentiviral vector doses, and analyzed 2 days later (on day +3 after cell stimulation) for GFP expression by flow cytometry (A) and microscopy (B). (A) Frequency ± SEM of transduced cells. n = 3–4. One sample t test versus 0, *$p < 0.05$. (B) Representative microscopy photos of CD8+ Treg culture transduced with 0.1 to 10 MOI GFP-lentiviral vectors, magnification ×10. (C) CD8+ Tregs were

(legend continued on next page)

the volume of the culture medium during the transduction may affect cell growth. Cytokines should be freshly added during T cell culture and stored as single-use aliquots at −80°C. Addition of rapamycin does not significantly reduce the transduction rate or cell growth in $CD8^+$ Tregs (Figure 5C).

Cell stimulation. Dose and timing of the stimulation are crucial for human $CD4^+$ and $CD8^+$ Treg culture (Figure 4). It should be noted that $CD8^+$ T cells require lower stimulation than what is usually recommended for $CD4^+$ T cells to survive. High stimulation can be deleterious for $CD8^+$ T cells, while low activation is mostly compatible with $CD4^+$ T cell culture. Stimulation once a week and after cell sorting is important for Treg growth. $CD8^+$ and $CD4^+$ Tregs can be exponentially expanded up to 4 weeks.[15] Sorting of human T cells using anti-CD3 mAb as indicated does not affect transduction rate or cell stimulation.

Cell density. Cell density inferior to 10^5 cells/0.32 cm^2 (96-well flat bottom plate) on day 0 seeding of human T cells may inhibit cluster formation during ON stimulation, while higher cell density may limit cell proliferation (Figure 5D).

Lentiviral vector dose. High doses of lentiviral vector may be deleterious for cell growth (Figures 3C, 3D, and 6C), and the dose should be balanced between transduction rate and cell growth. Removing lentiviral particles by washing human cells is not necessary.

Loss of function in genetically modified expanded cells

Culture medium supplements. Tregs and Teffs require different culture conditions. To preserve high cytotoxic activity of human Teffs, addition of 100 U/mL IL-2, 5 ng/mL IL-15, and 5 ng/mL IL-7 is recommended.[16,26] To preserve Treg function, addition of 1,000 U/mL IL-2 and 10 ng/mL IL-15 for $CD8^+$ Tregs only is recommended.[15] Addition of 50 nM rapamycin is recommended for preserving the regulatory function of human and mouse Tregs during culture.[15]

Anticipated Results

Human and mouse Treg isolation and CAR-Treg engineering

The use of a FACS Aria sorter allows isolation of Tregs with more complex combination of marker expression, including low expression, and results in a great purity (≥95%) of both human $CD4^+CD25^+CD127^{low/-}FOXP3^+$ Tregs and $CD8^+CD45RC^{low/-}$ Tregs (Figures 1A and 1B) and mouse $CD4^+CD25^+FOXP3^+$ Tregs and $CD8^+CD45RC^{neg}$ Tregs (Figures 2A and 2B). We generated lentiviral vectors with VSVg- and MLV-pseudotyped envelope proteins to transduce human and mouse cells, respectively. Those vectors encoded for the 2nd generation of CARs containing CD28 and CD3ζ signaling molecules and single chain variable fragment (scFv) of mAb specific to HLA-A*02, Human Epidermal Growth Factor Receptor-2 (HER2) (Figure 1C), or MOG (Figure 2C) and the ΔNGFR as reporter markers. Further isolation of transduced Tregs on reporter marker expression (fluorescent protein or ectopic surface marker) by FACS sorting is optional but provides a pure subset of genetically modified Tregs for straighter conclusions on downstream functional assessments (Figure 1D). Immunogenicity, toxicity, interference with cell function, or migration should be considered when choosing a reporter marker. The truncated ΔNGFR human protein is non-immunogenic and non-endogenously expressed on hematopoietic cells; thus, it can be used safely as a reporter marker compatible with clinical applications.[27] All manufactured HLA-A*02-CAR and MOG-CAR Tregs and untransduced cells expressed a high level of FOXP3 protein (Figures 1E and 2D).[15,16]

Determination of the optimal dose of lentiviral vector to obtain the maximum transduction rate in Tregs

To determine the optimal dose of lentiviral vector required for transducing Tregs, we tested a range of GFP lentiviral vector doses, from 0.2 to 20 MOI, on human $CD8^+$ Tregs and analyzed the GFP expression by flow cytometry and microscopy (Figures 3A and 3B). Two days after transduction, a dose-dependent GFP expression was observed in clustered cells by microscopy (Figure 3B) and was confirmed by flow cytometry, reaching a maximum GFP expression in $CD8^+$ Tregs transduced with 10 MOI of virus and tending to decrease to a lower rate of transduction when using a higher dose of lentiviral vector (Figure 3A). To improve the transduction rate in Tregs, we tested increments of one to four successive transductions of $CD8^+$ Tregs, $CD4^+$ Tregs, or total T cells at the rate of one transduction per day (Figures 3C and 3D). By cumulating up to 2 transductions of $CD8^+$ Tregs, we did not observe any significant improvement of the transduction rate or loss in cell viability based on eosin coloration, but we observed an important drop in cell growth correlating with four cumulated lentiviral vector doses (Figure 3C). In contrast, up to three successive transductions of $CD4^+$ Tregs or total T cells significantly increased both transduction rate and cell yield (Figure 3D). These results indicate that two transductions of $CD8^+$ Tregs and three transductions of $CD4^+$ Tregs or total T cells with a MOI of 10 of lentiviral vector are optimal to obtain the maximum of viable transduced cells.

Optimal Treg stimulation is critical for their transduction with VSVg-pseudotyped lentiviral vectors and expansion

T cell stimulation significantly promotes cell transduction with VSVg-pseudotyped lentiviral vector through upregulation of the LDL receptor.[28,29] Since that anti-CD3 and anti-CD28 mAb

transduced with a range of GFP-lentiviral vector doses on day 1 (blue triangles), days 1+2 (red triangles), days 1+2+3 (gray squares), or on days 1+2+3+4 (black crosses) and analyzed on day 7 after stimulation by flow cytometry for frequency (left) and growth ± SEM of transduced cells (right). Growth of transduced cells was calculated by dividing the number of GFP^+ cells harvested on day 7 by the number of cells seeded on day 0. n = 3. (D) $CD8^+$ Tregs (red squares, n = 3–6), $CD4^+$ Tregs (blue circles, n = 2), and total T cells (black triangles, n = 3) were transduced with 2 MOI of GFP-lentiviral vector on day 1, days 1+2, days 1+2+3, or on days 1+2+3+4 and analyzed on day 7 after stimulation by flow cytometry for frequency (upper left) and growth ± SEM of transduced cells (bottom left). Growth of transduced cells was calculated by dividing the number of GFP^+ cells harvested on day 7 by the number of cells seeded on day 0. Two-way repeated-measure ANOVA and Bonferroni post-test versus 1 transduction, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. Right: representative dot plots of GFP expression in T cell subsets.

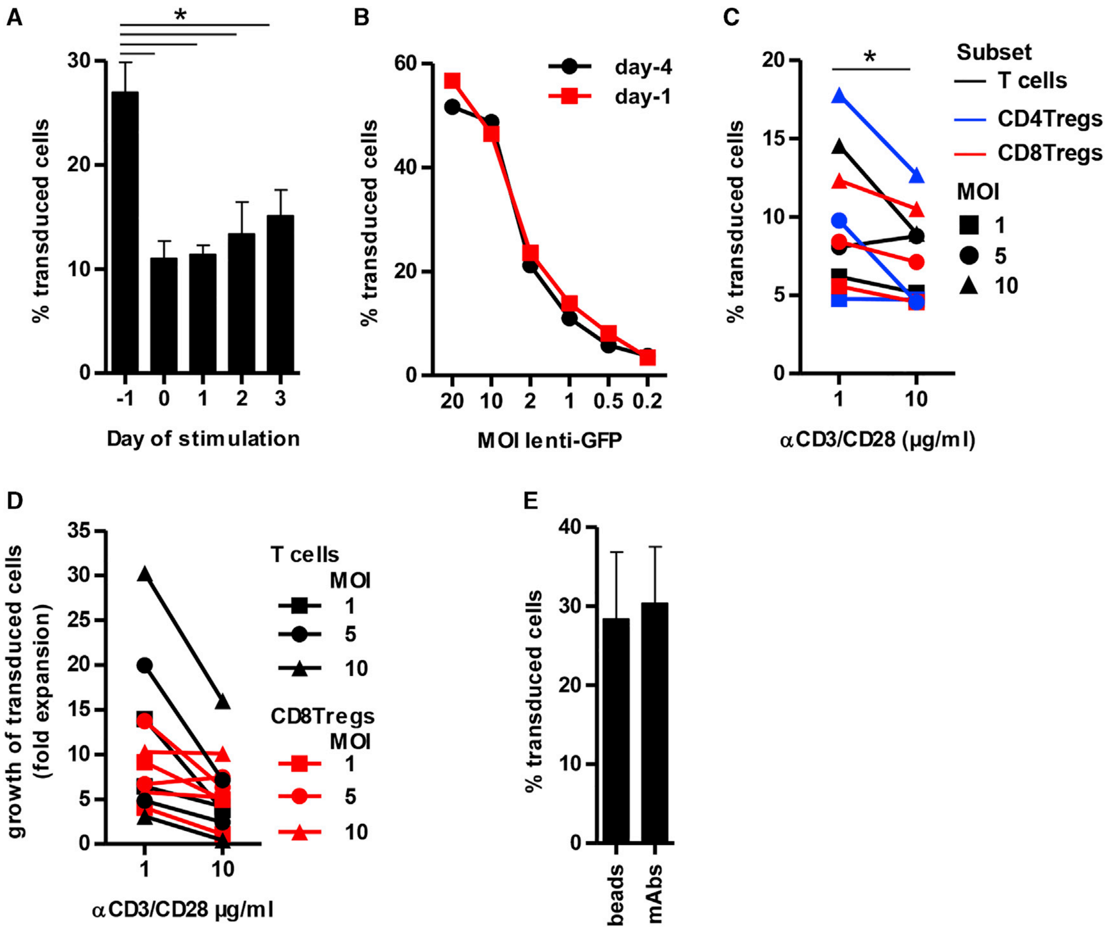

Figure 4. Optimal Treg stimulation is critical for their transduction with VSVg-pseudotyped lentiviral vectors and expansion

(A) $CD8^+$ Tregs were stimulated 1 day before, on the day of, or on days 1, 2, or 3 following transduction (day of transduction = day 0) and analyzed on day 8 by flow cytometry for transgene expression. Frequency ± SEM of genetically modified cells is shown. n = 4. Mann Whitney test, *$p < 0.05$. (B) Frequency of genetically modified $CD8^+$ Tregs analyzed on day 7 after cell stimulation during 1 (red squares) or 4 days (black circle) before transduction with a range of lentiviral vector doses. Two-way ANOVA test and Bonferroni test, ns. (C) T cells (black lines), $CD4^+$ Tregs (blue lines) and $CD8^+$ Tregs (red lines) were stimulated with a range of anti-CD3 and anti-CD28 mAbs ON, transduced twice with a 1 (squares), 5 (circles) or 10 (triangles) MOI of lentiviral vector doses, and analyzed on day 4 for transgene expression by flow cytometry. Frequency ± SEM of positive cells is shown. n = 1–5. Two-way ANOVA test, *$p < 0.05$ for 1 versus 10 μg/mL. (D) $CD8^+$ Tregs and T cells were stimulated with a low (1 μg/mL each) or high dose (10 μg/mL each) of anti-CD3 and anti-CD28 mAbs (day 0), transduced twice with a range of lentiviral vector doses on days 1+2, sorted on transgene expression on day 7, and cultured until day 14 as described in the procedure. Growth was calculated by dividing the number of purified genetically modified cells harvested on day 14 by the number of cells seeded on day 0. n = 2 for each condition. (E) Frequency ± SEM of genetically modified $CD8^+$ Tregs stimulated 1 day before 10 MOI lentiviral transduction with anti-CD3 and anti-CD28 mAbs (1 μg/mL) or GIBCO Dynabeads Human T-Activator CD3/28 (ratio beads:cells, 1:1) assessed by flow cytometry on day 8. n = 6, Wilcoxon matched paired test, ns.

stimulations are likely required to both expand and transduce Tregs and that transduction of the cells at the beginning of the amplification process would considerably reduce lentiviral vector consumption and costs, we investigated the optimal timing for stimulating and transducing Tregs. We stimulated $CD8^+$ Tregs either 1 day before, on the day of, or the days after the lentiviral transduction (Figure 4A). As expected, stimulation of the cells before transduction greatly improved lentiviral transduction compared to stimulation on the day of or after transduction (Figure 4A). In addition, stimulation 4 days or 1 day before transduction did not impact the transduction rate (Figure 4B). Then, we investigated the dose of anti-CD3/anti-CD28 mAb stimulation required for obtaining maximum transduction efficacy and transduced cell yield. As we previously set up a protocol to expand human $CD8^+$ Tregs for cell therapy with 1 μg/mL plastic-coated anti-CD3 and 1 μg/mL soluble anti-CD28 mAbs,[15] we explored the impact of a higher stimulation (10 μg/mL each mAb) on human $CD8^+$ and $CD4^+$ Treg transduction rate in a range of lentiviral vector doses (Figures 4C and 4D). We observed that a high stimulation resulted in lower transduction rates (Figure 4C) and was detrimental for the growth of transduced cells over 2 weeks of culture (Figure 4D). Finally, we considered using beads instead of purified mAbs to stimulate Tregs before lentiviral transduction and

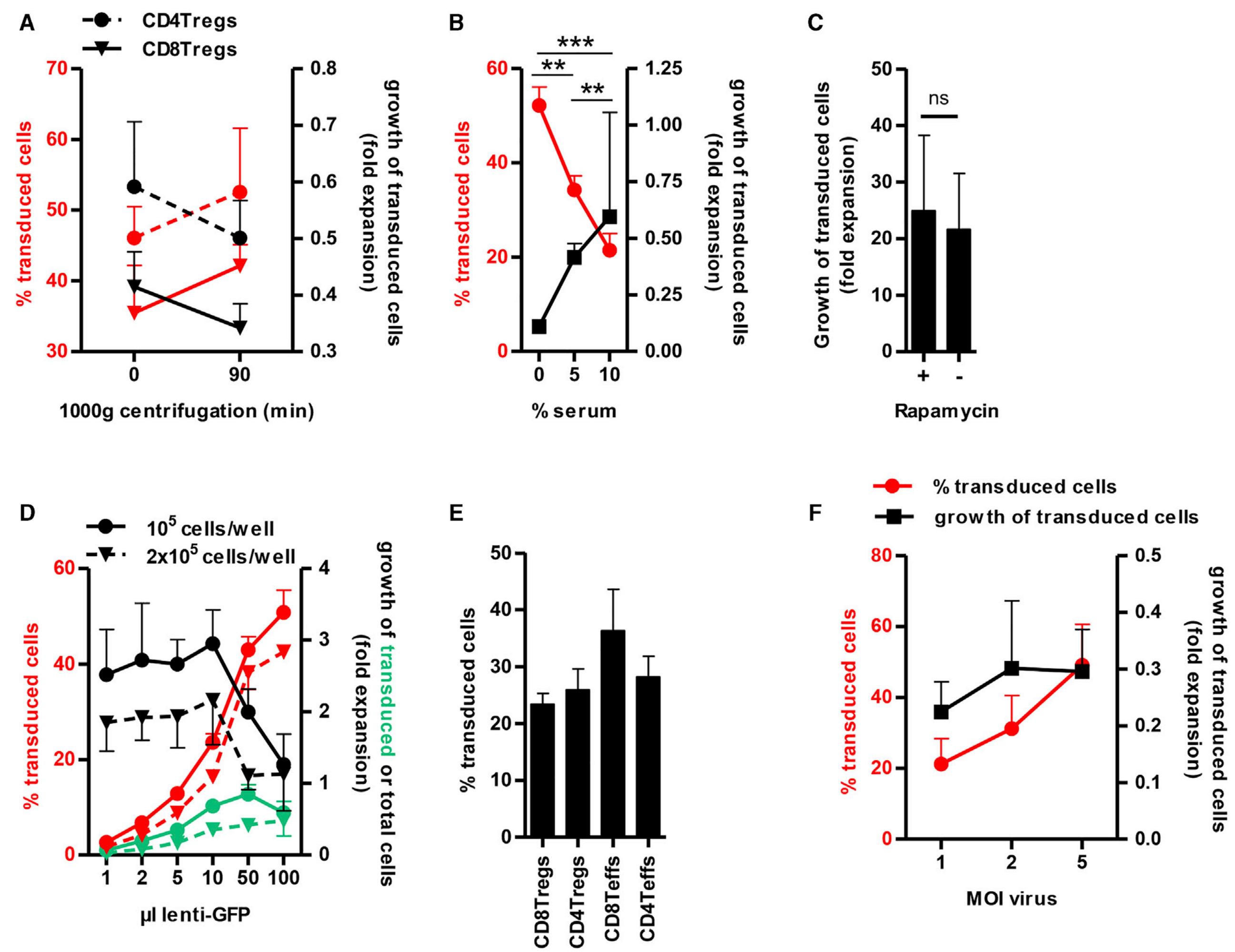

Figure 5. Culture parameters refinement for human Treg transduction and expansion

(A) Frequency (left y axis) and growth (right y axis) ± SEM of genetically modified CD4$^+$ (dotted lines) and CD8$^+$ Tregs (solid lines) 4 days after transduction with a range of lentiviral vector doses and with or without 90 min 1,000 *g* centrifugation. Cell growth was calculated by dividing the number of purified genetically modified cells harvested on day 4 by the number of cells seeded on day 0. n = 4. (B) Frequency (left y axis) and growth (right y axis) ± SEM of genetically modified CD8$^+$ Tregs 8 days after transduction with 10 MOI lentiviral vector in absence or presence of 5% or 10% human AB serum from day 0 to day 3. n = 4–21, Mann Whitney, **p < 0.01, ***p < 0.001. (C) Growth ± SEM of genetically modified CD8$^+$ Tregs transduced as described in the protocol and harvested after 14 days culture in presence or not of 50 nM rapamycin. Cell growth was calculated by dividing the number of purified genetically modified cells harvested on day +14 by the number of cells seeded on day 0. n = 6, Mann Whitney, ns. (D) Frequency (left y axis, red lines) and growth (right y axis, green lines) ± SEM of genetically modified CD8$^+$ Tregs and growth (right y axis, black lines) of total CD8$^+$ Tregs seeded at 10^5 (circles) or 2×10^5 (triangles) cells/well in a 96-well flat bottom plate and transduced with a range of GFP-lentiviral vector doses. Cell growth was calculated by dividing the number of genetically modified- or total cells harvested on day 7 by the number of cells seeded on day 0. (E) Frequency ± SEM of genetically modified CD4$^+$ and CD8$^+$ Tregs and Teffs transduced as described in the protocol after 7-day culture. CD8$^+$ Tregs, n = 54; CD4$^+$ Tregs, n = 32; CD8$^+$ Teffs, n = 9; CD4$^+$ Teffs, n = 3. 3 independent technicians. (F) Frequency (left y axis, red lines) and growth (right y axis, black lines) ± SEM of genetically modified CD8$^+$ Tregs thawed, stimulated, and transduced twice with a range of GFP-lentiviral vector doses. Cell growth was calculated by dividing the number of genetically modified cells harvested on day 7 by the number of cells seeded on day 0. n = 3.

obtained similar transduction rates (Figure 4E). Altogether, these results suggest that the optimal conditions to transduce CD8$^+$ or CD4$^+$ Tregs with a VSVg-pseudotyped lentiviral vector are a low (1 µg/mL purified mAbs or low dose beads) anti-CD3 and anti-CD28 stimulation 1 to 4 days before transduction. Using a specific antigenic stimulation (multimer, artificial antigen presenting cells [APCs] expressing the CAR target HLA-A*02) instead of polyclonal anti-CD3/anti-CD28 mAbs may privilege the expansion of the genetically modified clones.[12,28]

Culture parameters must be refined for human Treg transduction and expansion

To further increase Treg transduction rate and growth, we investigated transduction and culture parameters reported for Teffs. First,

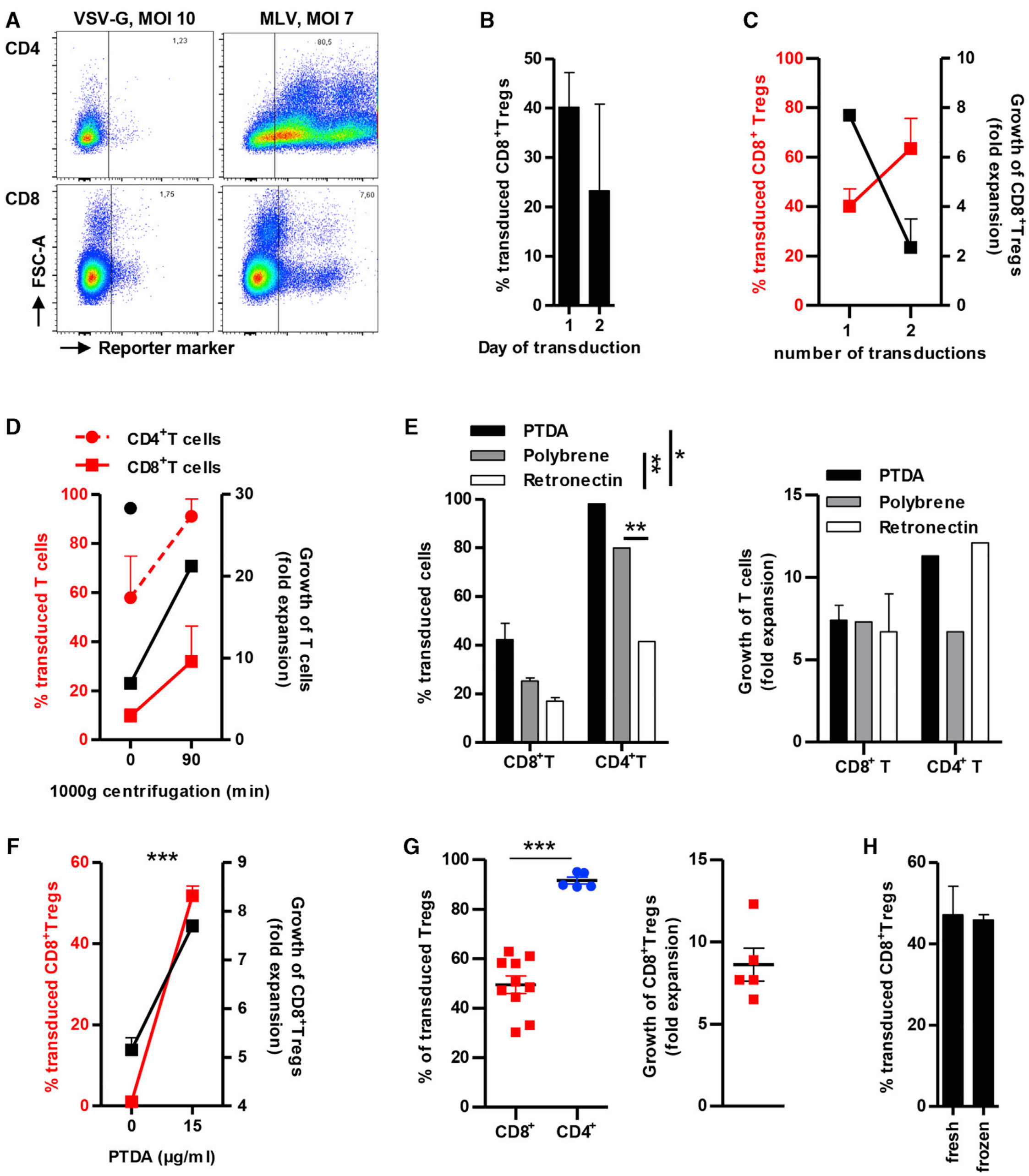

Figure 6. Mouse Treg engineering to investigate new engineered Treg therapy candidates *in vivo*

(A) Mouse CD4+ (upper) and CD8+ (bottom) T cells were sorted, stimulated ON with anti-CD3 and anti-CD28 mAb-coated beads, transduced with 10 MOI VSVg-pseudotyped (left) or 7 MOI MLV-pseudotyped (right) MOG-CAR lentiviral vectors, and analyzed for reporter marker (LNGFR) expression after 9-day culture. (B) Frequency ± SEM of genetically modified CD8+ Tregs after transduction at day 1 or day 2 after anti-CD3 and anti-CD28 mAb-coated bead stimulation. n = 2. (C) Percentage (left y axis, red line) and fold expansion (right y axis, black line) ± SEM of genetically modified CD8+ Tregs after transduction at day 1 or at days 1 and 2 after anti-CD3 and anti-CD28 mAb-coated bead stimulation. n = 2. (D) Frequency (left y axis) and growth (right y axis) ± SEM of genetically modified CD4+ (dotted lines) and CD8+ Tregs (solid lines) 7 days after lentiviral transduction with or without 90 min 1,000 *g* centrifugation. n = 1–4. (E) CD8+ and CD4+ T cells were transduced in the presence of PTDA, retronectin, or polybrene and analyzed after 6 days for the expression of ectopic protein (left) and fold expansion (right). n = 1–2. Two-way ANOVA and Bonferroni post-test, *$p < 0.05$, **$p < 0.01$. (F) Frequency of transduced cells (left y axis) and growth (right y axis) ± SEM of CD8+ Tregs transduced in the presence or absence of 15 µg/mL PTDA. n = 1–3. Two-way ANOVA and Bonferroni post-test, ***$p < 0.001$. (G) CD8+ and CD4+ Tregs were stimulated ON with anti-CD3 and anti-CD28 mAb-coated beads, transduced with MLV-pseudotyped lentiviral vector in the presence of PTDA and with 90 min 1,000 *g* centrifugation, and analyzed for ectopic protein expression (left) and fold expansion (right). n = 5–10. Mann Whitney, ***$p < 0.001$. (H) Frequency ± SEM of genetically modified CD8+ Tregs transduced with fresh or thawed supernatant of lentiviral vector production. n = 2–3. Mann Whitney, ns.

we assessed the benefit of a high-speed centrifugation to promote the contact of the lentiviral vectors with Tregs as described for CD4$^+$ T cells.[3,5,6,10] While 1,000 *g* spinoculation promoted lentiviral vector penetration into CD4$^+$ and CD8$^+$ Tregs, it was detrimental for Treg growth (Figure 5A). Composition of the culture medium may also be crucial for Treg transduction and growth. Indeed, our results show that supplementation of culture medium with human serum is required for high Treg growth; however, a high dose of serum significantly inhibited their transduction (Figure 5B). This may be due to the aggregation of viral particles in the presence of serum proteins or to the non-specific cross-reacting anti-VSVg Abs contained within human serum.[30] Thus, a low dose of serum (5%) during transduction and subsequent supplementation of culture medium are recommended. By contrast, rapamycin added for maintaining Treg function did not affect the transduction rate or the growth of transduced CD8$^+$ Tregs (Figure 5C). Finally, we assessed the issue of cell density during seeding. Interestingly, transduction of twice as many CD8$^+$ Tregs with the same dose of lentiviral vector did not significantly affect the transduction rate but decreased the growth of total cells, possibly through cytokine consumption, impoverishment of nutrients, or acidification of the culture medium (Figure 5D). Culture conditions reported for CD4$^+$ Treg transduction and expansion were not suitable for CD8$^+$ Treg culture. Notably, compared to CD4$^+$ Tregs, CD8$^+$ Tregs required lower anti-CD3/anti-CD28 mAbs stimulation and did not survive the addition of chemicals[3,10,11] or high speed centrifugation for the transduction step.[3,5,6,10] Setting up optimal conditions for CD8$^+$ Treg engineering and then applying it to other T cell subsets highlighted suitable conditions for both CD4$^+$ and CD8$^+$ Treg and Teff engineering with a VSVg-pseudotyped lentiviral vector (Figure 5E). Finally, we showed that CD8$^+$ Tregs can also be expanded, then frozen, thawed, stimulated ON, and efficiently transduced using this protocol to obtain a suitable yield for a cell therapy use (Figure 5F).

Mouse Treg engineering to investigate new engineered Treg therapy candidates in vivo

Mouse T cells are useful for proof of concept of new genetic engineering approaches and future T cell-based therapies. Thus, we set up a process to modify mouse CD4$^+$ and CD8$^+$ Tregs using lentiviral vectors. While Fransson et al.[9] reached 12% of transduction in murine CD4$^+$ T cells by using the pantropic VSVg-pseudotyped lentiviral vector, we barely obtained 0.5% to 1.5% transduced mouse CD4$^+$ and CD8$^+$ T cells using a dose of 10 MOI (Figure 6A). In contrast, the use of MLV envelope protein for lentiviral vectors has been reported as efficiently transducing murine cells and, in addition, is showing an interesting biosafety profile since it hardly infects human cells.[31] By using the ecotropic MLV-pseudotyped lentiviral vector, we obtained around 80% transduction in CD4$^+$ and 10% in CD8$^+$ T cells at comparable MOI doses (Figure 6A). As for human T cells, we investigated the optimal process for mouse Treg transduction (Figures 6B–6F). First, we observed that the first day following polyclonal stimulation, Tregs were more permissive for lentiviral transduction than the second one (40.15% versus 23.25%) (Figure 6B) and that the transduction rate after a second transduction was cumulated to the first (about 63.25%). However, cumulative transductions resulted in a drop in cell growth (Figure 6C); thus, one transduction is preferred. While 1,000 *g* spinoculation with lentiviral vector was detrimental for human T cell engineering (Figure 5A), it did not inhibit mouse T cell growth and even increased the transduction rate of genetically modified CD4$^+$ and CD8$^+$ T cells (Figure 6D). Then we explored chemicals reported to improve contact and transduction of T cells by retroviruses.[32] PTDA assembles into nanofibrils, which bind to viral particles and link with cellular membranes independently of a receptor. By bridging viruses to the cell surface, PTDA significantly improved the transduction rate in CD4$^+$ and, more particularly, in CD8$^+$ T cells compared to polybrene, which neutralizes the charge repulsion between the virus and cell membrane and to RetroNectin, a chimeric molecule binding virus to cell membrane dependently on VLA-4/VLA-5 without affecting their expansion capacity (Figure 6E). Importantly, we observed that PTDA was required for CD8$^+$ Tregs to be transduced by a lentiviral vector (Figure 6F). Finally, the protocol we set up results in 49.45% and 89.1% genetically modified CD8$^+$ Tregs and CD4$^+$ Tregs, respectively, with a great 8.62-fold expansion of mouse engineered CD8$^+$ Tregs (Figure 6G). Notably, this protocol is equally suitable for fresh or thawed lentiviral vector production supernatant (Figure 6H).

Discussion and Conclusions

This protocol describes the isolation of rare subsets of CD4$^+$ and CD8$^+$ Tregs, their transduction with lentiviral vectors, and expansion until obtaining high numbers of pure genetically engineered Tregs. By using this method, we generated HLA-A*02-specific CAR CD4$^+$ and CD8$^+$ Tregs that were highly efficient at preventing HLA-A*02-directed immune responses.[10–12,16] We previously showed that the CAR-CD8$^+$ Tregs were functional because target recognition by the CAR could activate T cells through CD28 signaling and persisted *in vivo* for at least 80 days for A2-CAR CD8$^+$ Tregs in HLA-A*02 transgenic mice[16] and 40 days for A2-CAR CD4$^+$ Tregs recruited into HLA-A*02$^+$ skin grafts.[10,11] Tools for cell isolation of CD8$^+$ Tregs, such as high purity grade anti-CD45RC mAb, is being developed,[33] and genetic modifications of Tregs for clinical application are being further developed within the Reshape consortium (http://www.reshape-h2020.eu). The second part of this protocol aims to identify and test novel targets in mice to enhance current cellular Treg products by describing our best process for engineering functional murine MOG-CAR CD4$^+$ and CD8$^+$ Tregs, which differs from human T cell engineering on aspects of both culture and transduction. Indeed, murine cells barely survive *in vitro* after 9 days of culture, whereas human Tregs can be expanded for up to 1 month *in vitro*; they are more efficiently transduced with MLV-pseudotyped lentivirus than with VSVg-pseudotyped ones; and the growth of genetically modified murine Tregs is hampered by cumulative transductions but promoted by chemical and mechanical helpers.

Lentiviral vectors are commonly used to deliver a transgene into T cells as efficiently or more efficiently than naked DNA and retroviral vectors.[21] Production of lentiviral vectors for clinical application can be expensive. Nevertheless, to reduce the cost of CAR-Treg manufacturing using lentiviral vector,[34] we propose the transduction of Tregs right after isolation and before expansion, thus reducing the amount of lentiviral vector required for genetically engineered Treg production.

Finally, this method is widely extendable to restore the expression of deficient genes;[35] stabilize, improve, or convert T cell functions through FOXP3 or IL-10-enforced expression, for example;[36,37] improve survival and *in vivo* persistence of cells through self-sufficient IL-2 production, for example; and confer a specificity, such as TCR[5,38] or guide migration (CAR[8]) of Tregs or Teffs. The method of mouse Treg isolation, culture, and transduction will be useful to identify and test new targets for proof-of-concept research studies.

ACKNOWLEDGMENTS

We thank Tuan Nguyen, Malika Gantier, and Laurent David for their advice regarding lentiviral vectors production. We thank Megan Levings and Sangamo Therapeutics France SAS for providing the CAR plasmids. Sangamo Therapeutics France SAS, formerly known as TxCell S.A., provided the protocol for mouse CD4$^+$ T cell transduction and partially funded this work. We thank P. Guerif, I. Guihard, and A. Fleury from the CHU of Nantes for their help in providing human blood. We thank Jenny Greig for English corrections in this article. We thank the Fondation Progreffe for financial support and Crédit Agricole for the donation of the FACS Aria. This project has received funding from the European Union's Horizon 2020 research and innovation program under grant agreement No. 825392 "RESHAPE". This work was also funded by the Labex IGO program supported by the National Research Agencyvia the investment of the future program ANR-11-LABX-0016-01 and the Agence de la Biomedecine. This work was realized in the context of the Institut Hospitalo-Universitaire (IHU)-Cesti project (ANR-10-IBHU-005). Labex IGO and IHU-Cesti projects are part of the "Investissements d'Avenir" French Government program managed by the ANR. The IHU-Cesti project is also supported by Nantes Metropole and Région Pays de la Loire. This work was also realized in the context of the support provided by the Fondation Progreffe.

AUTHOR CONTRIBUTIONS

N.V., J.L., and S.B. conducted the experiments; S.B. and C.G. designed the experiments; S.B. and C.G. wrote the paper; N.V., J.L., and I.A. revised the paper; C.G. and I.A. funded the experiments.

DECLARATION OF INTERESTS

S.B., I.A., and C.G. have patents. The remaining authors declare no competing interests.

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